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14. ABSTRACT

The focus of the Spellman/Gray work group over the past year has been upon the generation of materials, tools, and data for the purpose of aiding and supporting the research and findings of the entire multi-team collaboration endeavoring to identify antigenic targets for breast cancer-infiltrating T cells. Our team has achieved a number of accomplishments over the current funding year. We have characterized immunogenic peptides from a collection of MHC-I-bound epitopes eluted from the cell surface of several breast cancer cell lines. A computational pipeline was also developed to identify the sequence of the complete TCR heterodimer, working synergistically with data collected following single-cell emulsion RT-PCR. Additional modifications were made to our epitope discovery workflow to increase efficacy of transcript and neoantigen candidate prioritization for future research, and strides are being made in development of a personalized T cell-based protocol for identification of T cell-activating epitopes.

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INTRODUCTION:

The OHSU Spellman/Gray work group is one of three collaborators funded by this Department of Defense Breast Cancer Multi-Team Award; the other two being comprised of the Lee work group from City of Hope (formerly of Stanford Medicine Cancer Institute) and the Slansky/Kappler work group from University of Colorado Denver/National Jewish Health. The major objective of this endeavor is to develop novel strategies aimed at the enhancement of the protective effects of anti-tumor T cells *in vivo* in a patient-specific manner based on the hypothesis that partially protective anti-tumor T cells exist within TDLNs in most breast cancer patients. This will be accomplished by identifying the antigens anti-tumor T cells target in different breast cancer subtypes, potentially including antigens preferentially expressed by breast cancer stem cells. We will identify both MHC-I- and MHC-II-restricted antigens driving both CD8 and CD4 anti-tumor T cells *in vivo*, as CD4 T cells are needed to optimally sustain vaccine-elicited CD8 T cells *in vivo* [1]. Identified antigens will be categorized as to breast cancer subtype-specificity or shared status amongst subtypes, with the intention a patient could be matched with an optimal set of vaccine antigens for her tumor. Another novel aspect of this project is the identification of altered peptides (mimotopes) that may more efficiently activate anti-tumor T cells than the natural tumor epitopes. A final objective is to identify small molecule anti-cancer agents that synergize with cytotoxic T lymphocytes (CTLs) to enhance immune-mediated killing. Collectively, this undertaking will produce a set of immunologically validated antigens and mimotopes for major breast cancer subtypes, and a set of agents that cooperate with immune killing. These can be used in combinations in a patient-specific manner to maximize clinical benefit while minimizing toxicity. The tools we develop will enhance the breadth and efficacy of existing and future approaches for immune therapy of breast cancer. We discuss here the Spellman/Gray group's specific efforts toward realizing the goals of this collaboration.

KEYWORDS:

Breast cancer, cytotoxic T lymphocytes, RNAseq, MiTCR, immune response, epitopes

OVERALL PROJECT SUMMARY:

RNAseq analysis of tumor cells [Task7]

RNAseq analysis to identify breast cancer-specific aberrant transcripts. In our goal to identify breast cancer-specific antigens via systematic computational analysis, we continue to resolve errors and implement improved approaches to our analytical pipeline to ensure output of high-confidence epitope candidates. To biologically validate a subset of ten of the most highly ranked candidate transcripts via rtPCR, a cohort of ten tumor samples with matched normal tissue was acquired from the OHSU Knight Biolibrary. The cohort consists of two triple-negative tumors, two HER2-enriched tumors, and six ER-positive tumors.

As the predicted peptide sequence of a number of these candidates is the result of a single nucleotide change, sequencing of the aberrant region would be required for validation. Upon further inspection, the majority of the single nucleotide differences were determined to occur only at splice junctions, suggesting their prediction could be the result of erroneous alignment by Tophat, specifically alignment of 1-3 bases at the end of the read to the adjacent intron rather than to the correct position(s) at the beginning of the next exon. This issue has been addressed and corrected by more current spliced alignment programs, such as GSNAP, STAR, and Tophat2; however, the majority of our dataset was aligned with Tophat in early 2013, overlapping the release date of Tophat2.

Before committing laboratory resources to candidate validation, we have chosen to analyze a subset of the TCGA breast tumors using an alternative alignment algorithm (Spliced Transcripts Alignment to Reference [STAR]) and selecting candidates supported by both algorithms. Progress for this portion of the project was suspended for approximately 90 days of this funding year due to the physical relocation of our computational center followed by extensive upgrades to the cluster and servers. We are now aligning 20 TCGA breast cancer (BRCA) RNA sequences with STAR and will search assembled transcripts for members of our candidate subset. Candidates with support from both the Tophat and STAR algorithms will be validated in the laboratory.

Additional improvements to the pipeline involve the inclusion of a substantial number of additional RNA sequencs to the original set of approximately 700 breast tumors (TCGA) and nearly 370 normal tissues (TCGA,GEO,EBI). We are now incorporating RNA sequence data for additional >400 TCGA breast tumors, nearly 300 TCGA normal tissues, and >1700 GTEx normal tissues. The ongoing development of our normal tissue dataset continues to be a priority, as it provides a robust resource of normal transcript expression levels across all tissues against which tumors are compared for identification of uniquely expressed transcripts. With a dataset now more than double the original, along with the challenges encountered using an older alignment algorithm, we intend to run the complete dataset through the pipeline; however, it is imperative we reduce the computational resources required while improving alignment accuracy. To this end, we will implement STAR v2.4.2a for alignment to the most current version of the human reference genome (GRCh38), as STAR runs in a fraction of the time with similar alignment accuracy to that of Tophat2. The remainder of the pipeline will remain unchanged.

Separately we used tumors and normal tissue collected from our study to generate RNAseq data. Samles from 25 HLA-A2+ positive patients followed by total RNA purification and RNAseq analysis (exome sequencing). RNAseq libraries have been produced and have been analyzed by the OHSU sequencing core. The data are now being processed and will be used to identify overexpressed genes and mutations that can be presented in HLA-A2-loaded epitopes. RNAseq data will facilitate identification of immunogenic epitopes from cancer patients.

Generation and initial analysis of T cell clones [Task 5]
TCR sequencing of each clone. We have also continued to carry out analysis of sequencing data accumulated from the University of Denver's innovative emulsion rtPCR technique, which yields information regarding the identity of individual T cells through evaluation of paired TCR chains. High-throughput data analysis is being carried out using our modified version of miLaboratory's MiTCR TCR receptor repertoire analysis software, which we have coined CompleteTCR. As described in previous progress reports, the original software was adjusted to output clonotype diversity of paired alpha-beta chains, rather than diversity of only one chain. This combined effort has resulted in the discovery of a TCR pair shared amongst 15 of 20 patient tumors and a number of other TCR pairs shared by 7 or more tumors. Since the TCR is examined ex vivo, data is not skewed by the effects of cell culture or cell death, lending confidence the shared TCR pairs are tumor-specific and hold potential as targets for immunotherapy. Results of this study are being prepared for publication by the Denver team, and we will make the CompleteTCR software package and source code freely available to the research community under a GNU license via GitHub.

Statistical analysis was carried out by our team to determining the false discovery rate of shared clonotypes, comparing those shared amongst the 22 tumor PBMC samples to those shared amongst the 7 normal/control PBMC samples. Of interested, we found the majority of statistically significant TCRs shared between the normal/control PBMC samples were absent from all 22 tumor PBMC samples. Interpretation and implications of these results will be reported by the Denver team.

During the course of the grant we have also sought to support the other teams by identifying potential epitopes that could be used to analyze T cell clones. As a result we have worked on the characterization of epitopes eluted from breast cancer cellines. To identify immunogenic peptides we used T cell activation protocol published in Blood (2007) (1) and J Immunol Methods (2006) (2).

Briefly, dendritic cells (DCs) were generated from HLA-A2-positive peripheral blood mononuclear cells (PBMCs) by a 90-min incubation at 37 °C in DC medium. Non-adherent cells and media were removed and replaced with 1 ml/well of fresh DC medium supplemented with 1000 IU/ml GM-CSF and 1000 IU/ml IL-4. After one day of incubation, DCs were matured using 10 ng/ml lipopolysaccharide (LPS) in the presence of peptide (10 μg/ml). Next day, peptide pulsed DCs were irradiated (32 Gy) and mixed with autologous CD8+ T cells and incubated for 7 days. On the day 4, IL-2 (50IU/ml) and IL-7 (5 ng/ml) were added to the medium. This stimulation was repeated 3 times.

Secondary stimulation was set up as described above, except that artificial antigen-presenting C1R-A2 were used. This secondary stimulation was repeated 3 times. After final stimulation, T cells were harvested and used in INF-γ ELISA with C1R-A2 cells loaded with peptide. **Figure 1** shows that peptide N 147 activates T cells as was evidenced by increased secretion of IFN-γ by T cells in the presence of peptide as compared to T cells without peptide or in the presence of HLA-A2 blocking antibodies.

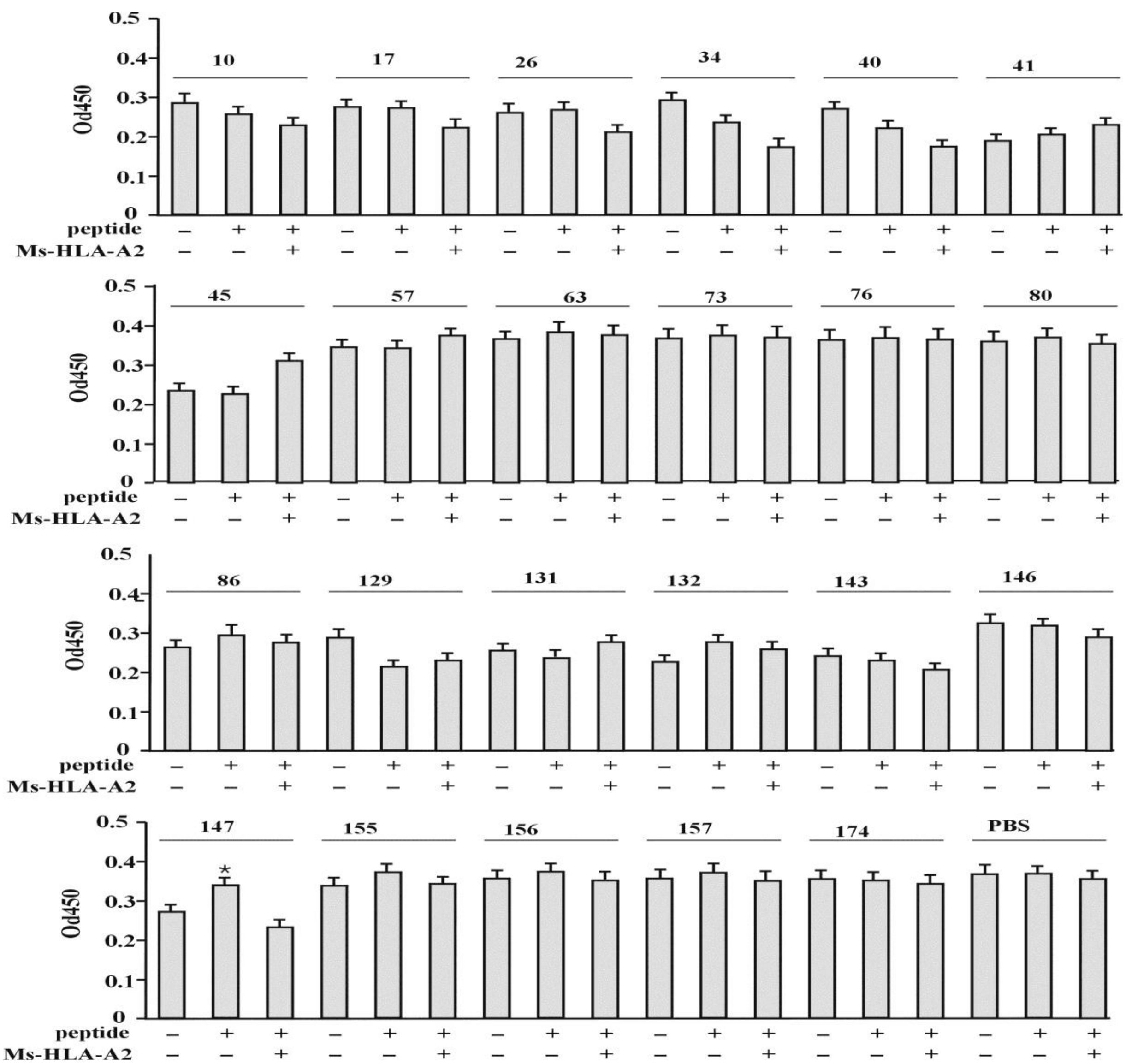

Fig. 1. **IFN-γ ELISA**. Plate was coated with IFN-γ capturing antibodies (1 μg/ml, 100 μl/well) in PBS overnight at 4°C. C1R-A2 cells (10^5/well) were added to wells and pre-incubated with Ms-HLA-A2 (BB7.2) blocking antibodies (10 μg/ml) for 1 h. Next, C1R-A2 cells were loaded with peptide (10

μg/ml) and incubated 2 h followed by addition of T cells stimulated with corresponding peptide. C1R-A2/T cells mixes were incubated for 24 h and IFN-Y secretion was analyzed by IFN-γ-biotin antibodies and avidin-HRP. Signal was visualized by TMB-E and 1N HCL. Absorbance at 450 nm was measured using ELISA reader. *, P = 0.05.

In addition, to determine if the eluted MHC class I-bound epitopes have been identified in previous studies we searched Immune Epitope Database (www.iedb.org) consisting of 27,413 human unique peptidic epitopes. We determined that of the 2822 eluted unique epitopes, 803 epitopes have been shown to bind MHC class I proteins in MHC class I ligand binding assays. Importantly, 9 and 1 peptides in our peptide data set were active in T cell and B cell activation assays, respectively (**Table 1**). In addition, we found that most of these immunogenic epitopes were not related to breast cancer and even two of them were exclusively targeted by T cells in chronic lymphocytic leukemia (CLL) patients (3). This analysis demonstrated both that established cancer cell lines can be used as a source for the identification of tumor specific and immunogenic epitopes and that immunogenic epitopes can be shared between different cancer types including leukemia and solid tumor cells.

Peptide sequence	Assay	Epitope source	MHC class I restriction	tumor type	Number of identifications	Reference
GLLGTLVQL	^{51}Cr release	Catenin β-1 [CTNB1] (400-408)	HLA-A*02:01	breast, ovarian, prostate	6	4
ALSDHHIYL	^{51}Cr release	Fructose-bisphosphate aldolase A [ALDOA] (216-224)	HLA-A*02:01	breast, ovarian, prostate, renal cell carcinoma	7	4, 5
SLFVSNHAY	^{51}Cr release	Fructose-bisphosphate aldolase A [ALDOA] (356-364)	HLA-B*15:02, HLA-A*3003	in vitro, B cell line JS	1	6, 7
SQFGGGSQY	^{51}Cr release	Eukaryotic translation initiation factor 3 subunit D [EIF3D] (61-69)	HLA-B*15:02; HLA-B*15:01;	in vitro; B lymphoblastoid cell line 721.221	2	6, 8, 9
NVIRDAVTY	^{51}Cr release	Histone H4 [H4] (65-73)	HLA-B*15:02	in vitro	0	6
VTAPRTLLL	^{51}Cr release	HLA class I histocompatibility antigen, B-37 alpha chain precursor [1B27] (3-11)	HLA-E	in vitro	0	10
VTAPRTVLL	^{51}Cr release	HLA class I histocompatibility antigen, B-50 alpha chain precursor [1B50] (3-11)	HLA-E; MHC class I(B) molecule Qa-1b	in vitro; H-2^{b} lymphoblasts	1	10, 11
ISDGPSKVTL	Immunoblot detection of antibody/antigen binding	Coilin [COIL] (257-266)		in vitro	0	12
LLDVPTAAV	Positive MHC: epitope complex binding to TCR	Gamma-interferon-inducible lysosomal thiol reductase [GILT] (27-35)	HLA-A*02:01	in vitro; breast, ovarian, prostate; hepatocellular carcinoma cells and solid tumors	4	4, 13-16
YGYDNVKEY	ELISPOT IFN-γ release	CDCA7L protein [CDA7L] (422-430)	HLA-A*03, HLA-C*03, HLA-C*12	chronic lymphocytic leukemia (CLL)	7 positive CLLs (23%)	3

Table 1. Immunogenic peptides that have been eluted from the cell surface of breast carcinoma cells.

The knowledge of the type of HLA-A, B, and C allele allowed us to predict the binding probability of each peptide to HLA allele present in the corresponding cells. For this propose, we used the

Consensus method (17) consisting of ANN (18, 19), SMM) (20), and Comblib (21). This choice was motivated by the high predictive performance of the Consensus method. The predicted affinity is expressed as an IC50 value. After that, we calculated a percentile rank for each peptide, which was generated by comparing the peptide's IC50 against those of a set of random peptides from SWISSPROT database. Smaller percentile rank indicated higher affinity of peptide. Then, we calculated a percentile rank of binding to HLA-A*02:02 allele for each peptide and plotted the number of peptides (counts) in relation to these percentile ranks (**Fig. 2**). Then, we selected cell lines that have homozygous HLA-A*02 genotype (MDA-MB-231, MCF7, and LY2), heterozygous HLA-A*02 genotype (HCC1419, HCC1428, BT549, CAMA-1, MCF12A, UACC812, and SUM159PT), and HLA-A*02-negative cells, and we plotted the peptides that were derived from these cells in relation to percentile ranks (**Fig. 3**).

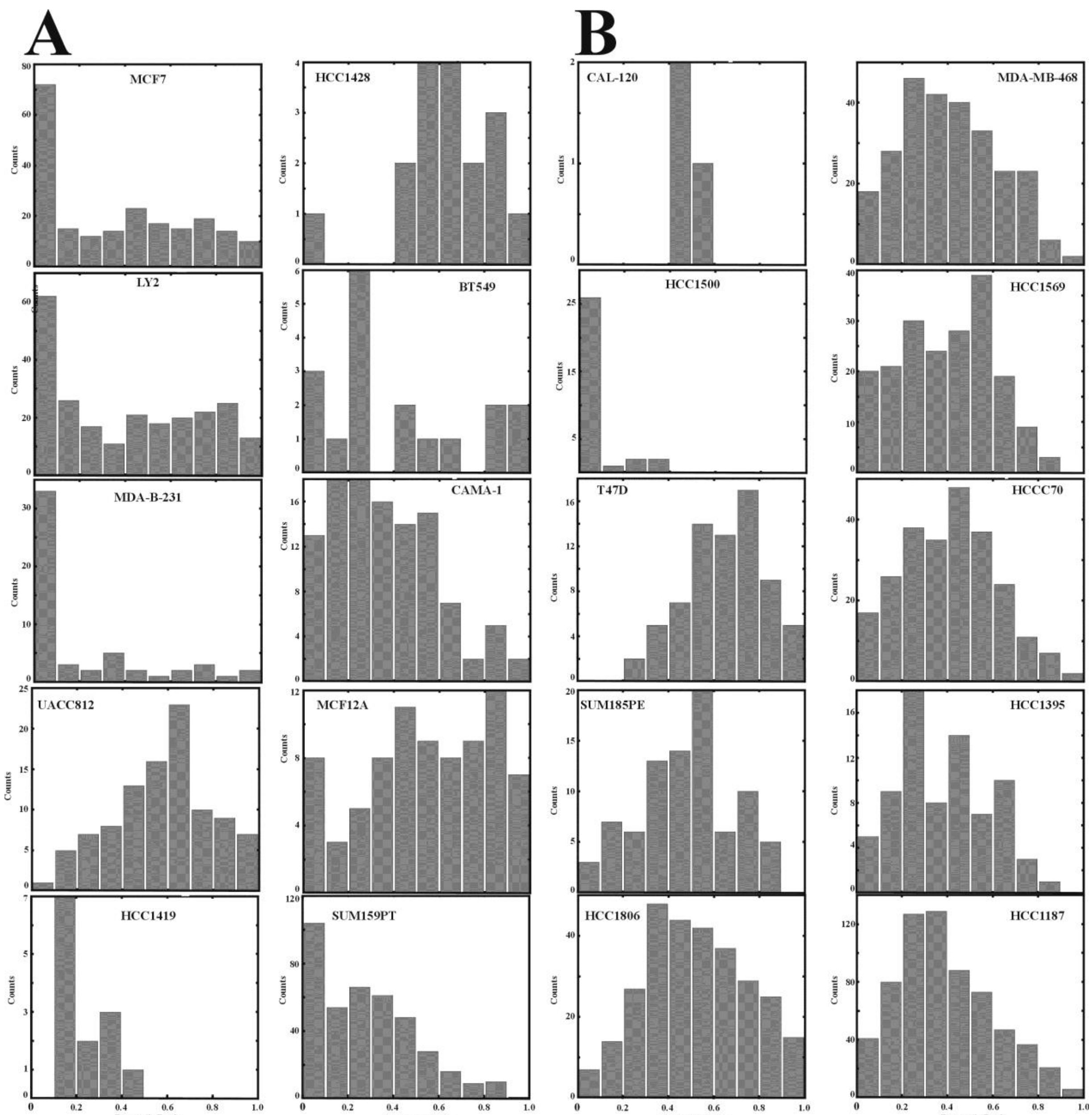

Fig. 2. The histogram of distribution of peptides according to HLA-A*02 binding score in genotypically HLA-A*02-positive (A) and –negative (B) cell lines.

To predict the distribution of HLA-A*02-specific peptides in breast carcinoma cells, we used a beta distribution mixture model (22). To fit the peptide distribution curve in our beta mixture model we used an Expectation Maximization (EM) algorithm (23) implemented in Python. The EM algorithm is a probabilistic model to find the distributions that best explain a set of data. It obtains the optimal parameters for the fitted distributions by maximizing the log-likelihood function. The likelihood function describes how well a set of parameters, in this case beta distribution shape coefficients and distribution proportions, explain the data.

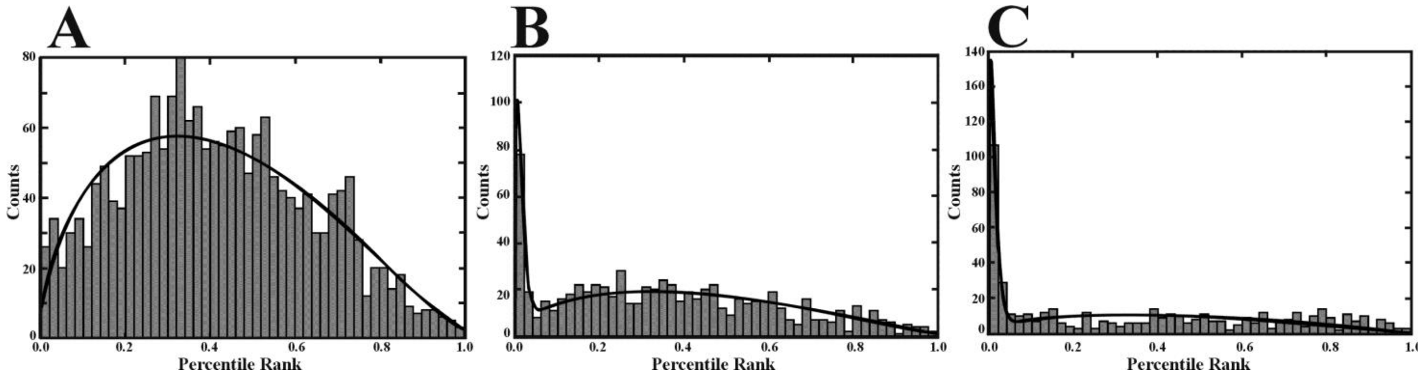

Fig. 3. The histogram of distribution of peptides in genotypically HLA-A*02-negative (A) and HLA-A*02-positive heterozygous (B) and homozygous (C) cell lines.

For HLA-A*02-negative cell lines, we modeled the data with one distribution to find the shape parameters for HLA-A*02-negative distribution. When modeling cell lines with an HLA-A2-positive allele, we maximized likelihood function to find the ratio between HLA-A2-positive and HLA-A2-negative alleles as well as the shape parameters for the HLA-A*02-positive distribution. The result from modeling the data was two beta distributions corresponding to HLA-A2-positive and HLA-A2-negative peptide distributions. In the follow up analysis we determined the most likely proportion of HLA-A2-positive peptides within each cell line from which we eluted at least 50 peptides (**Table 2**). We can envision that in the future to design personalized vaccine, MHC class I-bound peptides will be eluted directly from tumors. Based on the HLA allele types present in the same patient, it will possible to make distribution profiles for each allele type and predict what type of MHC class allele presents the most peptides.

Cell line	FDR	HLA-A*02-	HLA-A*02+
MDA-MB-231	0.08	0.53	0.39
LY2	0.05	0.72	0.23
MCF7	0.06	0.67	0.27
CAMA-1	0.01	0.91	0.08
MCF12A	0.01	0.91	0.08
SUM159PT	0.13	0.69	0.18
UACC812	0.02	0.97	0.01
HCC1187	0.06	0.91	0.03
HCC1395	0.09	0.90	0.01
HCC1569	0.06	0.89	0.05
HCC1806	0.06	0.93	0.01
HCC70	0.09	0.87	0.04
MDA-MB-468	0.06	0.92	0.02
SUM185PE	0.02	0.96	0.02
T47D HER2+	0.01	0.99	0.00

Table 2. Ratio of HLA-A*02-positive and -negative peptides in breast carcinoma cell lines.

KEY RESEARCH ACCOMPLISHMENTS:

- Performed follow up experiments on epitopes from MHC pulldowns to observe immunogenicity.
- Worked to analyze complex TCR data with Denver group.
- Determined that several epitopes discovered in MHC pulldown experiments have been previously identified and many have been found to immunogenic.

- Developed the ability to determine which MHC allele epitopes are being presented on in our MHC pull down experiments.

CONCLUSION:
The focus of the Spellman/Gray work group over the past year has been upon the generation of materials, tools, and data for the purpose of aiding and supporting the research and findings of the entire multi-team collaboration endeavoring to identify antigenic targets for breast cancer-infiltrating T cells. We have identified a number of candidates in breast cancer tissues as well as breast cancer cell lines, utilizing a variety of analytical methods. The epitope discovery pipeline is proof of concept of *in silico* epitope discovery from RNAseq data. It aids in the definition of the protein-epitope relationship by enlarging the knowledge base of protein-encoding transcripts beyond the protein models existing in public databases and by restricting the analyses to only the expressed transcripts. The results produced by this pipeline along with the MHC-I-bound epitopes identified by mass spectrometry in breast cancer cell lines will be used to rank epitopes for further characterization and development as therapeutic targets.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:
Manuscripts describing these results is in preparation.

INVENTIONS, PATENTS, AND LICENSES:
No inventions, patents, or licenses to report.

REPORTABLE OUTCOMES:
None

OTHER ACHIEVEMENTS:
No other achievements to report.

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APPENDICES:

No appendices to report.